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Cooperative transient trapping of protons by structurally distorted chloroplast ATPase: Evidence for the proton well?

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The structure of membrane-bound ATPase, CF_0CF_1, was distorted by mild EDTA treatment, but without solubilization of CF_1. Distorted CF_0CF_1 rapidly trapped protons from the lumen side without transport. Trapping required electric driving force, showed very high cooperativity as function of pH and hysteresis as function of time. It is discussed under the concept of a 'proton well'.

Introduction

The proton-translocating ATPase in thylakoid membranes of chloroplasts, CF_0CF_1, belongs to the ubiquitous class of F-type ATPases with bipartite structure. The extrinsic portion, CF_1, binds nucleotides and phosphate. When dissociated from its intrinsic membrane counterpart, it exposes CF_0, a proton channel with unparalleled selectivity. CF_0 discriminates protons against other cations from pH 6 to 8 and even in the presence of 300 mM KCl in the suspending medium. This has been evaluated by flash spectrophotometry [1]. In such experiments both photosystems are stimulated for single turnover by a short flash of light. This generates a transmembrane voltage (by 30–60 mV more positive in the lumen) and a pH difference (about 0.05 units more acid in the lumen) [2]. Three observables have been monitored, namely: (a) pH transients in the lumen, (b) transients of the transmembrane voltage, and (c) pH transients in the suspending medium [1,3,4]. When more than 50% of total CF_1 is solubilized by treatment of thylakoids with some 100 μM EDTA, the extent and the time-course of proton intake from the lumen and of charge transfer across the membrane are equal [1]. Proton binding by CF_0 and transfer across the membrane dielectric appear to occur in synchrony. The same holds for the coupled transfer of protons during ATP synthesis through the holoenzyme, CF_0CF_1 [5].

We report here on highly cooperative proton trapping by CF_0CF_1 without concomitant transfer of charges across the membrane. This effect is elicited by treatment of thylakoids with very low concentrations of EDTA, say 10 μM. It was first described several years ago [6]. The experimental results of the previous work were fully reproduced in this work. Further data led to better understanding of the mechanism of this effect, which is sketched in this article.

Materials and Methods

Thylakoids were prepared from spinach or pea by standard procedures [1], except for the absence of magnesium in the media (destacked thylakoids). The ionic milieu in preparation and storage media was dominated by 10 mM NaCl. The procedure to evoke transient trapping of protons was as follows. Concentrated stock (2–3 mg chlorophyll/ml) was diluted in distilled water to yield a chlorophyll concentration of 10 μM, the pH was adjusted to 7.5 at room temperature. EDTA was added, typically at 10 μM (for necessary variations, see below). After 2 min, NaCl was added at 10 mM to stop exposure to low ionic strength. Longer incubation and higher concentration of EDTA resulted in CF_1 solubilization with the drastic increase of the proton conductance by exposed CF_0. After preincubation, methyl viologen (10 μM) was added as electron acceptor.

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Abbreviations: DCCD, N',N'-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NTA, nitrolotriacetic acid.

The thylakoid suspension was excited with short flashes of light (saturating, 10 ms duration) at a repetition period of 10 s. Absorption changes were recorded by weak measuring light, which was gated to overlay the actual sampling interval plus a forerun of 300 ms. Cuvette pathlength was 2 cm. The transmembrane voltage was measured by electrochromic absorption changes of intrinsic pigments around 520 nm. pH transients in the thylakoid lumen were detected by measuring the absorption changes of added neutral red (13 μM) at 548 nm in the presence of a non-permeating buffer and subtracting the absorption transients recorded plus and minus added dye [1,5].To improve the signal-to-noise ratio, 20–40 transient signals were averaged. The appropriate EDTA concentration to yield proton trapping without too much CF_1 solubilization, and thereby proton conduction, had to be determined for each batch of leaves. It varied between 5 and 40 μM, probably following variations of the intrinsic contents of divalent cations of in the starting material.

Results and Discussion

Fig. 1 shows voltage transients (left) and pH transients in the lumen (right) for thylakoids that had undergone mild EDTA treatment. The traces read as follows: one flash of light electrically charges the thylakoid membrane in less than 1 ns, and causes proton release into the thylakoid lumen. Proton release is kinetically biphasic, it rises in less than 1 ms due to water oxidation and in about 10 ms due to plastoquinole oxidation. To kinetically label events attributable to CF_0, transients were recorded twice, with and without venturicidin (1 μM), a blocker of proton conduction through F_0F_1 and F_0 [1,7]. While the transient acidification of the lumen was diminished by pretreatment with EDTA, the decay of the transmembrane voltage was not. The effect on the pH transient was reversed by venturicidin (Fig. 1), by organo-tins and DCCD (data not shown). These agents are inhibitors of the ATPase and its proton channel [7]. Only for DCCD has a side effect on Photosystem II been observed [8]. Therefore, the diminished acidification of the lumen, reversible by specific inhitors of CF_0, was not attributable to diminished *proton production* but rather to *transient trapping of protons* at the lumenal side of CF_0CF_1 or of CF_0.

The lower traces in Fig. 1 show the respective differences between the pair of traces at the top. Trapping was very rapid as evident from the trace in the lower right of Fig. 1 (note: time-scale 5 ms). The seemingly selective disappearence of the rapid phase of proton release into the lumen has previously been interpreted to indicate selective capturing of protons from water oxidation [6]. However, trapping was also apparent when Photosystem II (and water oxidation) was blocked by addition of DCMU and when proton release was entirely caused by cytochrome b_6f/Photosystem I (data not shown). A different chelator of divalent cations, namely NTA, caused the same results.

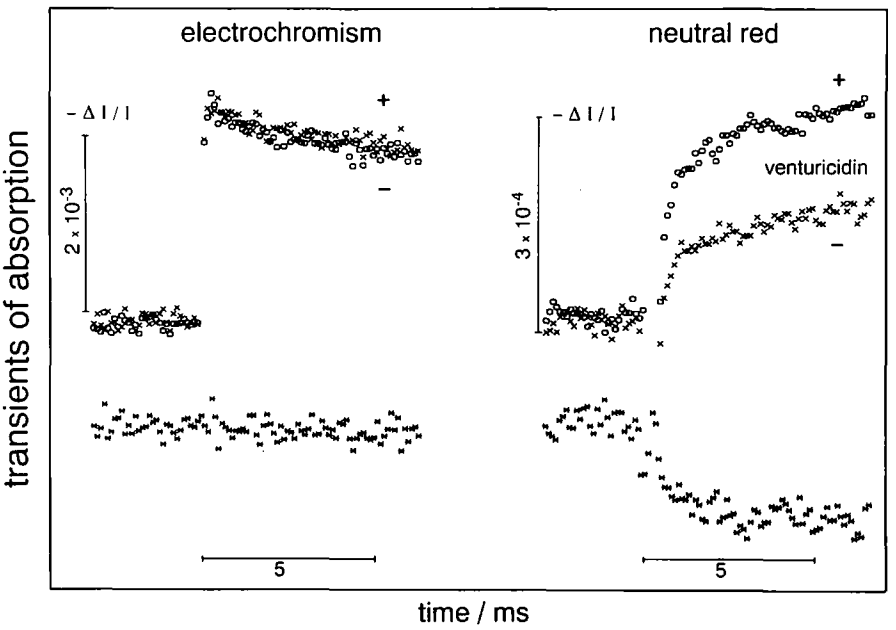

Fig. 1. Absorption changes at 522 nm indicating voltage transients (left) and at 548 nm indicating pH transients in the thylakoid lumen (right) in spinach thylakoids submitted to mild EDTA treatment (see above). Ordinate: relative transmitted intensity; abcissa: time. Venturicidin added when indicated (1 μM). Lower traces: difference between each pair of traces in the top. Assay mixture: chlorophyll, 10 μM; methyl viologen, 10 μM; NaCl, 10 mM; BSA, 2.6 g/l (pH 7.7). Digitizing interval: 17 ms, every fourth point displayed.

moved? Addition of very low amounts of divalent cations (a few μM) reversed transient trapping (data not shown). At these low concentrations of cations and with the very low concentration of possibly solubilized CF_1, rebinding of CF_1 to CF_0 does not occur. Hence, trapping was due to (distorbed) CF_0CF_1.

The difference between pH transients that were measured in the absence and in the presence of venturicidin (see lower left of Fig. 1) gives the time-course of

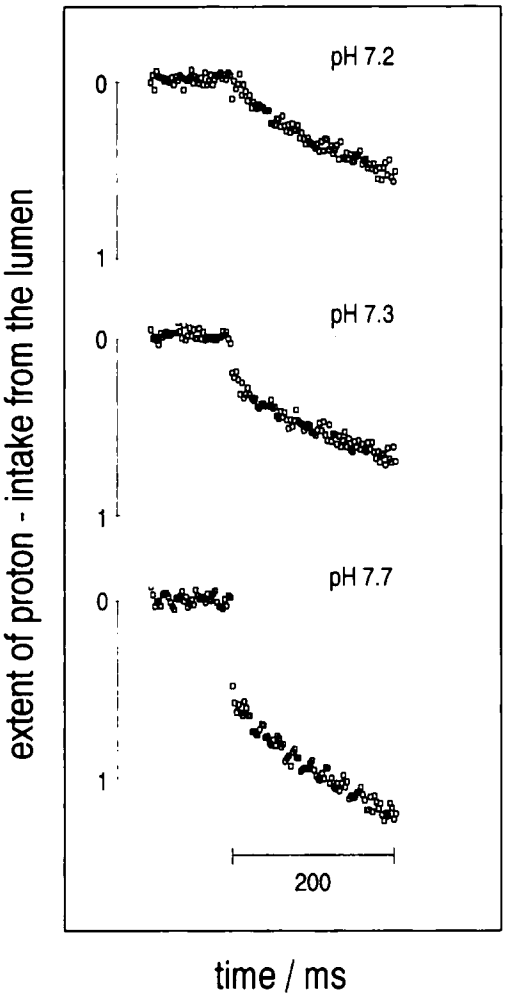

Fig. 2. Differences of pH transients in the lumen recorded with and without added venturidin. The transients indicate venturicidin-sensitive proton intake from the lumen of thylakoids after mild EDTA treatment. They were obtained under the same conditions as documented in the right column of Fig. 2 except for the pH, which is indicated in the figure. The ordinate scale was normalized to give number of protons taken up from the lumen per Photosystem II (see text).

CF_0-related proton intake from the lumen. This was recorded as a function of pH in the medium. In Fig. 2 the ordinate scale was normalized to indicate *the number of protons trapped per Photosystem II* (PSI : PSII, approx. 1 : 1). The maximum extent of the absorption transient (200 ms after the flash) in the presence of venturicidin served as standard. It was taken as 2 units at each pH. Thereby the pH dependence of indicator sensitivity and buffering capacity of the lumen was taken into account [11]. The traces at pH 7.7 were interpreted as follows: very rapid *proton trapping* was followed by much slower *proton efflux* (note different time scales, when comparing Figs. 1 and 2). Both were eliminated by venturicidin (see above). While the pH dependence of proton trapping was extremely steep (pH 7.7 was almost saturation level), the extent and rate of subsequent proton efflux (mediated by few exposed CF_0) was almost independent of pH in this narrow range. A fit of the extent of trapping by a cooperative binding isotherm ('fraction of unprotonated trapping groups' = 'total' $\cdot (([H^+]''/K'') + 1)^{-1}$) indicated hexacooperative behaviour (Hill coefficient $n = 6$) [6].

The pH difference which is generated by a single turnover of both photosystems is very small, about 0.05 units [11]. What, then, is the driving force of proton trapping? Fig. 3 shows pH transients in the lumen for thylakoids exposed to mild EDTA treatment in the presence (right) and in the absence (left) of valinomycin. The K^+-carrier valinomycin was added to accelerate the decay of the electric potential difference across the membrane from a half-decay time of about 50 ms to less than 1 ms. Valinomycin acted on both the rapid trapping of protons and the slower intake. It was evident from the traces in Fig. 3 that added valinomycin eliminated rapid proton trapping to the same extent as the CF_0-blocker venturicidin. This gave evidence that proton trapping was due to electric driving force. Added valinomycin also virtually eliminated the slower proton intake due to proton conduction through exposed CF_0. When measured over a longer time period it was not abolished but slowed down more than 10-fold, The reason for this (field-free relaxation of the buffering capacitance of thylakoids) has been given elsewhere [3].

The elimination of proton trapping by valinomycin required concentrations that accelerated the decay of the transmembrane voltage down to less than a millisecond half-decay time. If a second flash was fired, say 40 ms after the first one, proton release into the lumen was normal, without trapping. Only after a pause of some milliseconds were the trapping groups discharged and ready for another trapping act. Thus proton trapping seemed to be driven by electric force and it showed hysteresis in time. Hysteresis is common to systems with cooperative behaviour.

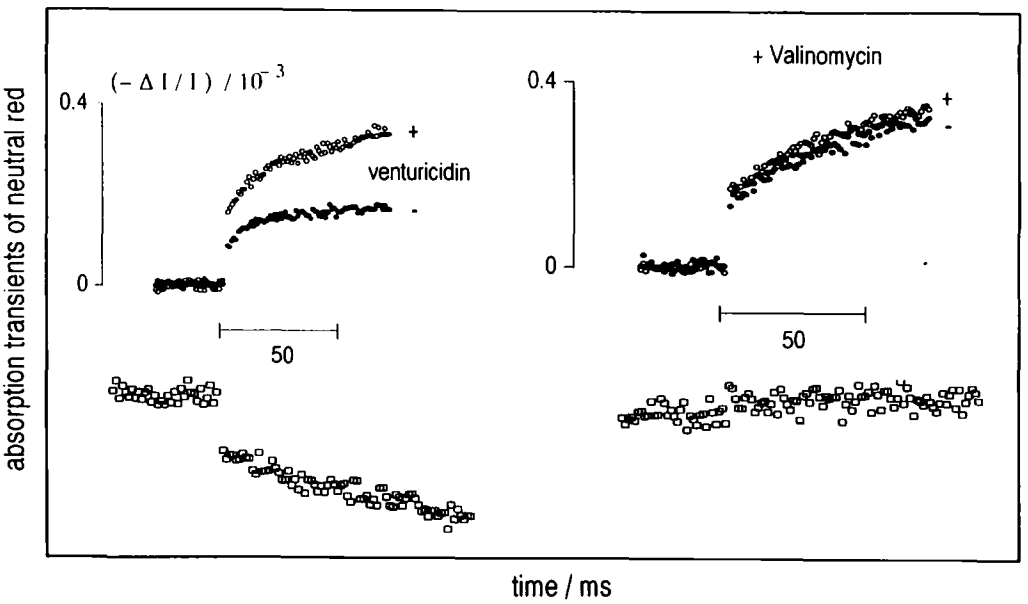

Fig. 3. Absorption changes of neutral red indicating pH transients in the lumen in the presence/absence of venturicidin Conditions as in Fig. 1, except for KCl, 20 mM instead of NaCl present. Left: without, right: with added valinomycin, 300 nM. Lower traces are difference between pair of upper traces, as in Fig. 1.

Within the limits of this short communication it is not possible to present more detailed experimental evidence for the interpretation that follows. Let it stand as speculation, though. We interpret the above results in the framework of Mitchell's proton well [12]. Assume a proton-specific domain that reaches from the lumenal surface of the membrane into CF_0CF_1. The lumenal surface still represents an *electric* equipotential surface, but the *electrochemical* equipotential surface stretches out into this proton-conducting domain. Potential buffering groups at its top experience a pH which is more acid than the one at the lumen surface. The pH decrease is equivalent to the voltage drop between lumen and the location of these groups in the membrane. This concept for the transformation of electric into chemical (entropic) force could account for the observed kinetic equivalence of $\Delta\varphi$ and ΔpH for ATP synthesis [13,14]. Without a proton well the kinetic equivalence is not at all trivial. Proton trapping is highly cooperative. In spinach thylakoids the pK of the trapping groups is 7.3 and the Hill coefficient around 6. This behaviour is characteristic of at least six identical and strongly interacting groups. The best candidates are two essential acid/base residues, Glu-61 and Arg-41 (see [15,16] for *E. coli*) on subunit III. Only this subunit of CF_0 is present in many copies (9–12 in *E. coli*) and derivatization of a single copy blocks ATPase activity (in *E. coli*, [17]).

Transient trapping of protons has only been observed with deranged CF_0CF_1. Is the trapping state an intermediate of the operational cycle of this enzyme? In the same type of flash spectrophotometric experiments as above, trapping has not been detected during ATP synthesis by CF_0CF_1 [5] or proton leakage through exposed CF_0 [1]. In both cases the respective transporter has carried out multiple turnovers. Each experiment was, however, an average over very many non-synchronous enzyme cycles. Time resolution of partial reaction steps was not expected. Thus, it is conceivable that mild EDTA treatment stabilizes, and thereby exposes to observation, one particular intermediate of the normal operating cycle of the ATP synthase.

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